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VIABILITY OF EMBRYONIC CHICK TISSUES FOLLOWING STORAGE AT LOW TEMPERATURES[1]

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(Received for publication on July 1, 1944)

Following earlier studies (Waterman, '39) on the survival of refrigerated tissues of 13- and 15-day rabbit embryos in omental grafts, it seemed of interest to investigate the viability of embryonic chick tissues after refrigeration by transplantation to the chorioallantoic membrane of the chick. The present study involves the use of chick embryos at various stages of incubation, low refrigeration temperatures, and storage of whole embryos for short and prolonged periods of time. It deals also with tissue survival and recovery, the question of differential sensitivity between rabbit and chick tissues, response of the transplanted tissues, general graft structure, and with the effects of refrigeration on the appearance of the donor tissues. This information may be helpful in determining the time limits of survival of embryonic tissues of warm-blooded animals.

It is well known that in warm-blooded vertebrates there exists a close correlation between temperature and normal development. The results of the rabbit embryo studies (cf. Nicholas, '34, '38, for the rat) show that mammalian embryonic tissues will survive refrigeration (above freezing), and that different tissues and organs vary in sensitivity to cold and storage (Luyet and Gehenio, '40; Alvarez, '37) and hence to anoxia. This demonstrated variation may contribute towards an explanation of the process of death of the embryo cultivated at subnormal temperatures (Needham, '42). Subnormal temperature alone is apparently not an immediate cause of tissue death. Nicholas (loc. cit.) and Waddington and Waterman ('33) have shown that rabbit and rat embryos will tolerate wide limits of temperature variation. In general, chilling becomes injurious or lethal only after having exerted its action on embryonic tissues for a relatively long period of time. The exceptions include brain, spinal cord, and notochord

[1]Financial assistance from the 1900 Fund of Williams College is gratefully acknowledged.

which are affected by even one or two days of storage. Fazekas and Himwich ('43) have pointed out that the ability of the adult mammal to withstand anoxia is much more limited than the infant.

Culture in vitro has been the principal method of testing the survival of embryonic chick tissues after chilling. Bucciante ('31) and Hetherington and Craig ('39) have shown that embryonic chick tissues remain viable for some time after refrigeration, and that certain tissues and organs are more susceptible than others to the abnormal conditions. In their review of the literature dealing with life and death at low temperatures, Luyet and Gehenio ('40) mention that fertile hen's eggs will survive storage at low temperatures for many days and that the embryos of birds were always found more resistant to low temperatures than the adults although almost nothing is known of the time at which the resistance decreases.

Materials and Methods

Chick embryos of 4- to 16-days incubation were removed aseptically from egg and extra-embryonic membranes, placed in sterile petrie dishes between layers of absorbent cotton moistened with Ringer's solution, and stored in refrigerators set at 0°, 5°, and 10° C. Thereafter, one embryo from each culture dish in the 5° and 10° refrigerators was transferred daily to fresh Ringer's solution at room temperature, and small pieces of its tissues and organs transplanted to the chorioallantoic membrane of 7- to 9-day chick embryos. The hosts were then returned to the incubator. At 0° C. the moistened absorbent cotton eventually froze as well as the film of moisture on the surface of the embryo, and after about 72 hours' exposure the smaller embryos were slightly rigid. Before transplantation, these embryos were first placed in cold Ringer's solution (10°-15° C.) and then transferred to fresh Ringer's solution at room temperature. It was impractical to attempt the isolation of organs of the youngest embryos, with the exception of eye, ear region, and limb buds, and hence small portions of these stages were used as transplants.

The method of chorioallantoic transplantation is well known and will not be described here. It is sufficient to mention that the chorioallantoic membrane of the developing chick is easily accessible for transplantation, and it provides the transplant with an adequate blood supply with relatively little pressure and congestion. Small trans-

plants become incorporated quickly by the host membrane and vascularization occurs by way of invading proliferations of host blood vessels. With the exception of the youngest stages mentioned above, the material transplanted included isolated rudiments or portions of kidney, brain, different regions of the digestive and respiratory tracts, liver, pancreas, eye, limb buds, skin, heart, gonads, etc. Since the presence of blood in stored tisues is said to aid survival (cf. Alvarez, loc. cit.), the loss of blood was avoided in the older embryos by tying the body stalk, which included the yolk sac and allantoic blood vessels. The length of the refrigeration period varied between 1 and 22 days.

The host eggs were examined seven to eight days after transplantation. At this time the grafts were removed and special note was made of their size and color. The grafts were fixed in Bouin's or Zenker's solutions, sectioned at 10-15 micra, and the sections stained with alum haematoxylin and eosin. The control for these experiments consisted of chorioallantoic grafts of fresh tissues of similar incubation ages.

Experimental

1. *Effects of Refrigeration on Donor Tissues*

Visible changes in the appearance of tissues and organs of embryos refrigerated at 5° C. appear gradually for the most part, and then only after relatively long storage periods. After 75 hours refrigeration most tissues of an 11-day chick embryo exhibit no appreciable structural change when compared with fresh tissues. There are several exceptions. The cells of brain, spinal cord, and notochord appear slightly shrunken due possibly to some dehydration; many of the nuclei are pycnotic and there are minute spaces scattered abundantly throughout the tissues. These changes point to a relatively early stage of degeneration. After transplantation, the tissues show little or no power of recovery. Other sensory tissues, e.g., ganglia, peripheral nerves, and larger sense organs, are more resistant to the injurious effects of refrigeration than brain and spinal cord. Further growth and differentiation of the donor tissues do not take place during the storage period and no mitotic figures are found in histological preparations.

Longer periods of refrigeration provoke more extensive injury and other more resistant tissues are progressively affected. Figure 10 is

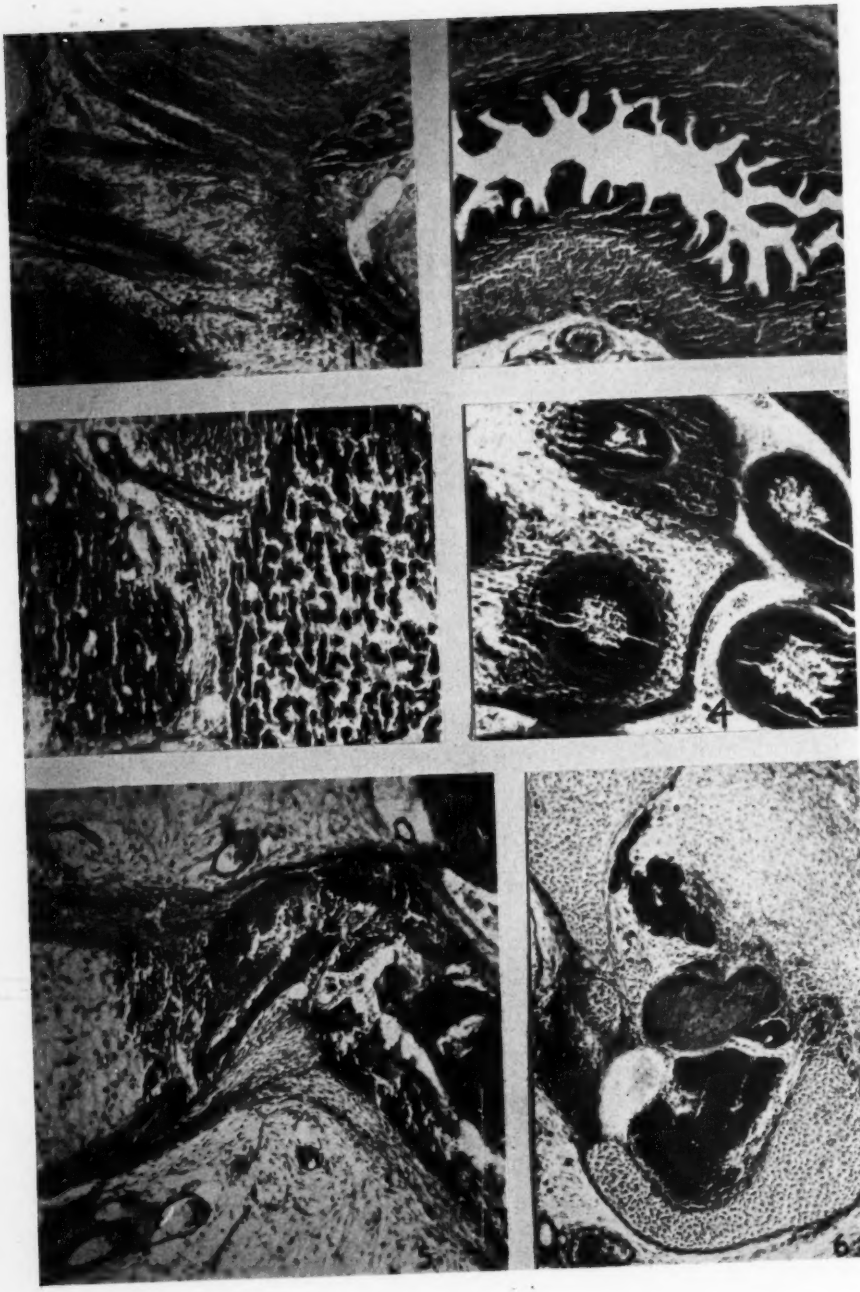

PLATE 1

(Figures 1-6)

(All figures are photomicrographs)

1. Portion of a longitudinal section through an 8-day chorioallantoic graft of a 96-hour limb bud (H18a2) showing skeletal muscle. x70. Previous to transplantation the donor embryo had been stored in a refrigerator at 5° C. for 10 days.
2. Section of the intestine in a 7-day graft (H10b3) from a 9-day chick embryo refrigerated four days. x100.
3. Liver (right) and pancreas (left) from a graft (H10b2) of material from the same donor as Figure 2. x70.
4. Section of developing feather primordia in a 7-day graft (H11a5) of skin from a 5-day donor refrigerated 11 days. x100.
5. Developing cartilage bone in a 7-day graft (H12h4) of the hind limb from a 13-day embryo refrigerated for four days. x100.
6. Section of an 8-day eye graft (H15a2) from a 4-day donor refrigerated 12 days. x70. A distorted lens appears in the center of the photomicrograph. Above it is a small mass of heavily pigmented tapetum nigrum and below it a heavily stained, folded retina bordered by a nodule of cartilage.

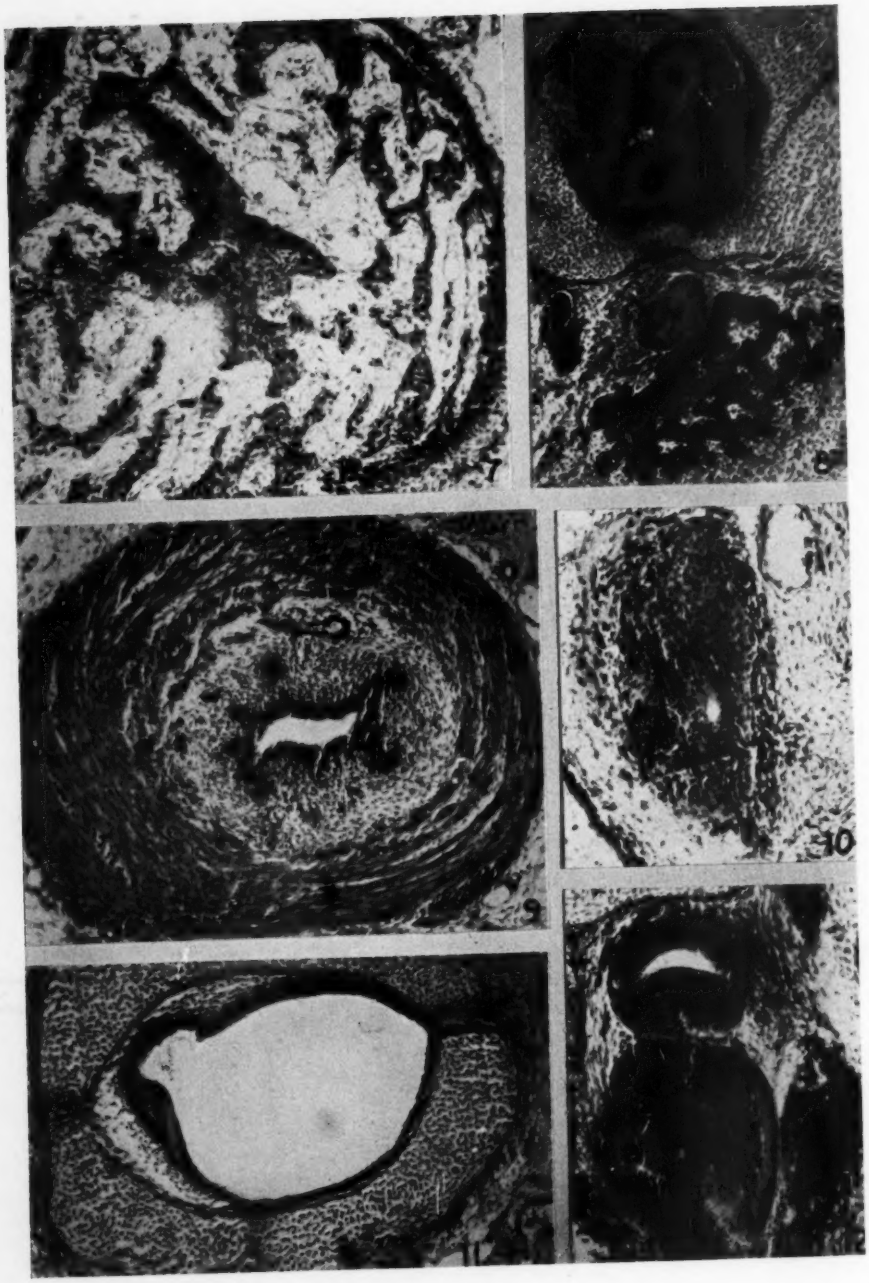

PLATE 2

(Figures 7-12)

7. Developing membrane bone from a 7-day graft (H12b3) of a portion of the head region of a 13-day embryo refrigerated four days. x100. Total age of tissue 24 days.

8. Section of an 8-day graft (H13a8) of the eye of a 4-day embryo refrigerated five days. x70. In the upper part of the photomicrograph is a mass of heavily stained retinal tissue bordered by cartilage. Betweeen this and the folded, heavily pigmented tapetum nigrum is a small lens.

9. Cross section of a 7-day graft (H10b2) of the intestine of a 9-day embryo, refrigeration four days. x70.

10. Portion of a section of an 8-day graft (H13a8) of the spinal cord of a 4-day donor, refrigeration five days. x100.

11. Section of the inner ear (8-day graft, H13a10) from a 4-day donor refrigerated five days. x70. The section includes a sensory area and the lower end of a vertical semi-circular canal. Cartilage surrounds the labyrinth.

12. Section of an 8-day graft (H18a2) of the eye from a 4-day embryo, refrigeration 10 days. x100. A small mass of heavily pigmented tapetum nigrum lies at the extreme right of a larger mass of retinal tissue. No lens was present in the graft.

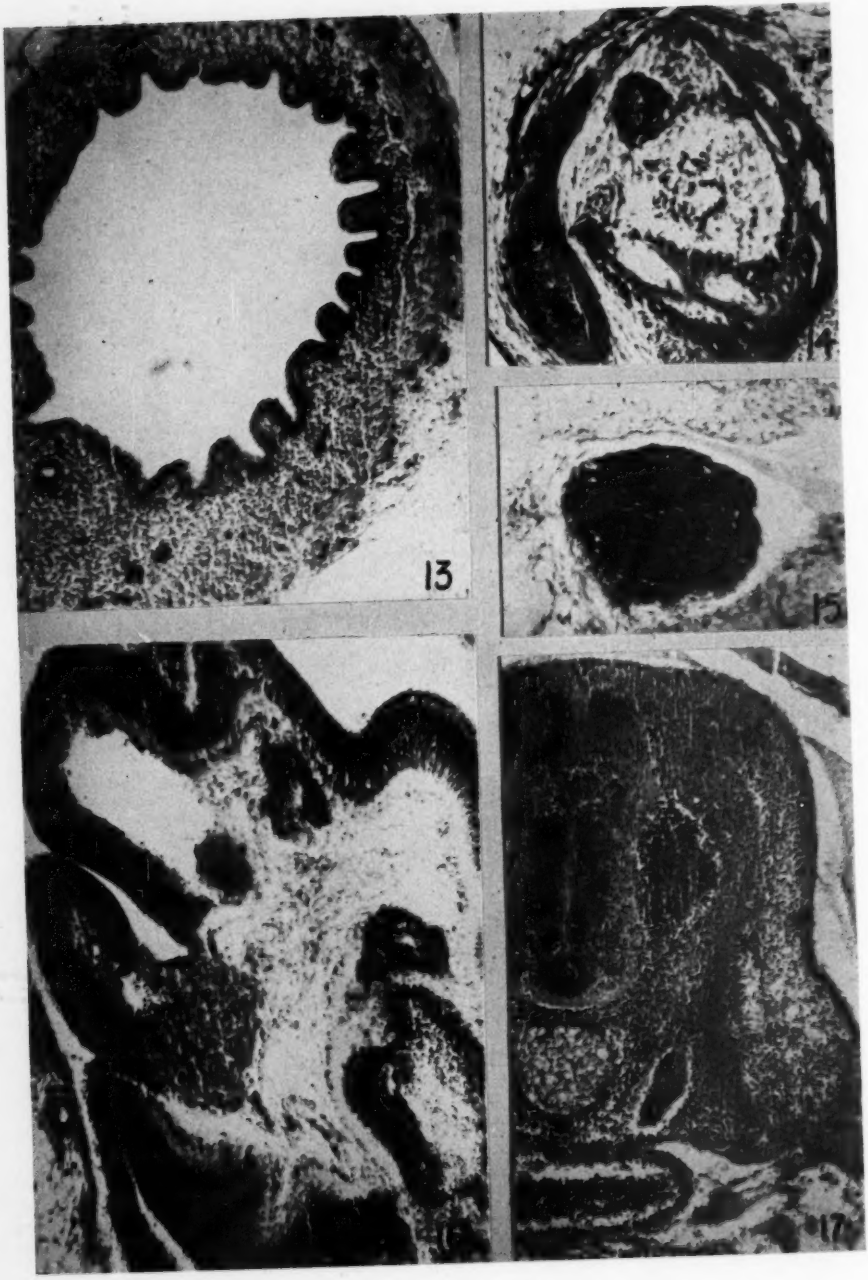
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PLATE 3

(Figures 13-17)

13. Section of an 8-day graft (H13a5) of the intestine from a 4-day donor, refrigeration five days. x100.

14. Developing cartilage bone and a small mass of epidermis in a 7-day graft (H10c5) from the fore limb of a 9-day embryo refrigerated for four days. x100.

15. Section of an indifferent gonad from an 8-day graft (H13a12) of a portion of the trunk region of a 4-day embryo refrigerated five days previous to transplantation. x70.

16. Section of the retina from a 7-day graft (H12f5) of the eye of a 13-day donor, refrigeration four days. x100.

17. Portion of a cross section in the region of the spinal cord and notochord of a 4-day donor embryo after 21 days of refrigeration. x70.

a section of an 8-day graft (H13a8) of the spinal cord and adjacent tissues from a 4-day embryo refrigerated for five days previous to transplantation. This is one of the few grafts of nervous tissue ever recovered. Its presence shows some attempt at recovery on the part of the transplanted spinal cord, but there is no resemblance to typical spinal cord structure. Tissues of a 7-day donor embryo fixed after six days refrigeration show degenerative changes in brain, spinal cord, and especially notochord. No change can be detected in the other tissues outside of some slight cellular shrinkage in muscle.

Figure 17 illustrates the change brought about in tissues of a 4-day chick embryo following 21 days refrigeration. The embryo maintained apparently normal form, but all types of tissues and organs show atypical cellular appearance and degenerative results of such a long storage period, e.g., markedly shrunken cells, pycnotic nuclei, innumerable number of minute spaces among the cells, and the frequent disappearance of the clear-cut boundaries between cells and different adjacent tissues. The structure of the few small grafts obtained from transplants of this material indicates that for the most part degeneration of the donor tissues had continued rapidly after transplantation. Only small bits of skin, a few feather rudiments, hyaline cartilage, cartilage bone and some skeletal muscle were ever recovered. The presence of small areas of advanced degeneration in some 8-day grafts points to short but futile attempts at recovery on the part of some of the original transplants. Sections of a 16-day embryo refrigerated for 22 days show a comparable type of injury to the cells. No grafts were obtained from transplants of this material in these experiments; all of the transplants being quickly resorbed. Possibly this negative result points to an increase in sensitivity with age and onset of function. For a discussion of this and suggested causes of death at low temperatures, reference may be made to Luyet and Gehenio ('40).

The best temperature for storage of whole chick embryos over long periods is approximately 5° C. The following range of temperatures was studied: 0°, 5°, and 10° C. It is well known (cf. Luyet and Gehenio, loc. cit.) that the internal body temperature of animals exposed to subnormal temperatures decreases slowly with time. This explains the numerous large grafts obtained with tissues refrigerated at 0° for 12 to 48 hours and the poor and even negative results ob-

tained with tissues at this temperature for much longer periods. Luyet and Gehenio state that some ice can be formed in embryonic tissues without killing them but the solidification of a large proportion of their water becomes lethal.

When the embryos were kept at 10° C. better results were obtained than at 0° C., but only for short storage periods of a few days. The large grafts contain a variety of surviving tissue, and the presence of areas of advanced degeneration point to some early attempt at recovery on the part of others. Progressively longer storage at this temperature produced more extensive injury and cases of successful recovery rapidly decreased in number. The results obtained with refrigeration at 5° C. are described in the following pages.

2. *General Graft Structure*

Although microscopic study of the donor embryos reveals certain structural changes, there is no perceptible change in external appearance of these embryos even at the end of 22 days of storage. Body outline is smooth, color unchanged, and the superficial blood vessel pattern distinct. Results found in the grafts may be taken as indicating the capacity of survival and the viability of the stored embryonic material. Small, white or reddish, well incorporated and vascularized grafts were obtained from all stages of development and periods of storage, but the size and number declined in the case of the material stored for the longest periods. This decline was found to coincide with the failure of certain tissues to survive the longest storage periods. Cyst formation is more common in the older grafts and in grafts of embryos after long refrigeration. These cysts are filled with fluid which coagulated in the fixing solution, and they are generally lined with gut or skin epithelium.

3. *Description of Grafts*

Information regarding incubation age of the donor embryos, length of refrigeration, number of grafts studied, and a brief summary of results are included in Table 1. For convenience in description and to limit repetition as much as possible, the experimental cases have been divided into six groups on the basis of the length of the refrigeration periods.

a. Ectodermal derivatives. Transplants of whole or pieces of

TABLE 1

Group	Cases	Days of incubation	Days of refrigeration	No. of grafts	Brief summary of results
1	H24, 25	4	3	12	Few small grafts of mesonephros, no metanephros. Brain tissue occasionally present. Spinal cord and ganglia more resistant. Spleen and notochord never found. Histogenesis of eye typical and retarded but not morphogenesis. Internal ear generally of vesicular type. Other tissues comparable to fresh tissue transplants.
	H4, 5, 7	7	2, 3	21	
	H1, 3	11	3	17	
	H9b, 2b	12	3	14	
2	H17	3	6	7	Representatives of most of transplanted tissues recovered. Three small pieces of brain tissue. Grafts large as in Group 1 above. No kidney and notochord. Necrotic and degenerating areas common within grafts. Fewer cases of disorganized spinal cord. Occasional grafts of gonad from youngest donors. Parts of multiple organs of disproportionate size.
	H13	4	5	5	
	H8	6	4	11	
	H9a, 10	9	4, 5	8	
	H12	13	4	7	
3	H16	3	9	12	More resistant tissues survived and grafts are smaller in size. Occasional bits of spinal cords, ganglia and pancreas. No brain tissue, heart, notochord, liver and kidney.
	H28, 29	5	8, 9	11	
	H30, 31	10	8	15	
	H19, 26	14	9	7	
	H21, 23	16	9	15	
4	H18, 15	4	10, 12	12	Grafts few in number and small in size. No spinal cord and pancreas. All ear grafts of simplest vesicular type. Few necrotic areas.
	H11, 22	5	11	13	
	H14, 20	9	11	9	
5	H2, 2a	3-5	15-17	11	Results comparable to Group 4 above. Marked increase in the amount of unidentifiable epithelial-like tissue. Increased disorganization among organ transplants.
6	H6, 6a, 27	3-7	21-22	14	Only epidermis, feather follicles, cartilage, bone and some skeletal muscle present in the few very small grafts recovered.

cerebral hemisphere and cerebellum, depending upon the size of the donor embryo, are found in grafts of Group 1 (cf. Table 1) which includes embryos refrigerated for two to three days. Even 24 hours refrigeration provokes disorganization and degeneration changes in these tissues that become intensified and more widely spread with longer refrigeration. Occasionally nervous tissue is recognizable in grafts of Group 2, but the amount is small, differentiation greatly retarded and identification uncertain in some instances. Blood cell

extravasation, phagocytosis and leucocyte infiltration contribute to disturbance of form and regional organization. It is well known that the nervous system is one of the first of the body tissues to show the effects of vascular disturbance. In general, transplants of any tissue that was fatally injured are so quickly removed by graft phagocytes that little, if any, trace remains in 7 to 8-day grafts. If recovery was initiated but not completed, only unrecognizable areas of degeneration are usually found or degeneration is well advanced. Thus this interval of time is sufficient to test viability. The presence of recognizable brain tissue indicates a weak recovery capacity aided possibly by a fortuitous location upon the chorioallantoic membrane. As far as could be determined, age and hence the state of differentiation of the stages tested did not appear to affect survival time.

Spinal cord is slightly more resistant than brain tissue to the abnormal conditions of the test medium as it is found occasionally in grafts of Group 3. However, few grafts were obtained even after much shorter refrigeration periods, and, like brain tissue, most show varying stages of degeneration and disorganization. Figure 10 is an example of the latter condition from an 8-day graft of spinal cord from a 4-day embryo refrigerated five days. The tissue is completely enclosed by a ring of cartilage. A few ganglia are present in the grafts. In general, transplanted tissues of the older stages tested are more typical in appearance than those from younger stages when the refrigeration period is short.

Figures 6, 8, and 12 are photomicrographs of eye transplants from 4-day donors refrigerated for 12, 5, and 10 days respectively. The presence of mitotic figures point to healthy growing tissues. Both eye and internal ear primordia are distinctly more resistant to refrigeration for long periods of time than are the tissues of the central nervous system. Figure 16 shows a portion of the retina in a 7-day graft of the eye of a 13-day donor refrigerated four days. Convoluted heavily pigmented tapetum nigrum, thickened retina and malformed lens are usually present in the eye grafts, although any one or two of the three parts may be missing. Whereas histogenesis has progressed in a typical, although retarded, manner, each type of tissue is more or less isolated from the others. In spite of the fact that whole eyes from the younger embryos were transplanted, no grafts exhibiting typical morphogenesis were found nor is there any recog-

nizable trace of cornea, optic nerve, or choroid and sclerotic coats. This is in contrast to transplants of fresh eye primordia which, although usually distorted due to tensions and pressures within the graft, may be quite typical in the structural relationships of the different parts of the eye. Eye tissues occur in grafts of even Group 4.

In all eye grafts the sensory retina is thick, generally folded, and frequently of a disproportionately large amount in comparison with other parts of the eye (Figure 16). Its histological structure is usually typical. Much connective tissue may lie between the folds or the folds may be tightly pressed together into an irregularly-shaped mass. Any differentiation into lenticular and sensory zones is not apparent. The thin layer of tapetum nigrum is likewise folded and generally is found as a smaller mass at one side of the retina. Its heavily pigmented condition makes it difficult to determine the number of cellular layers present. A small lens may lie between or to one side of the other tissues of the eye. Its general structures may be typical, including a layer of anterior epithelium and the nucleus of lens fibers, but the cells of the nucleus are irregularly enlarged and often of huge size. The lens appears to be the most resistant of all the eye tissues and may persist when everything else has disappeared from a graft. Eye grafts are usually bordered by one or more elongated masses of cartilage.

Many grafts of the internal ear from 3- to 6-day donors were obtained. Figure 11 is a section of an 8-day graft from a 4-day donor refrigerated five days. It is a cystic type of ear, small in size, and with two outpocketings projecting from the wall. Probably because of the presence of an enclosing wall of cartilage, the transplanted ear shows less distortion than that observed in eye grafts. One of the projections extends through a break in the cartilage wall and is identified as the endolymphatic duct. Its distal end is folded. The presence of a sensory area at the base of the other projection may indicate that the latter represents one of the semicircular canals. Two other sensory areas are present in the membranous wall. Many of the ear grafts from all stages tested are of this cystic type showing regional differentiation of the membranous epithelium, one or more sensory areas, diverticulum of one or at most two semicircular canals, and occasionally the diverticulum of the endolymphatic duct.

In the most completely differentiated ears there is a distinction

between sacculus and utriculus, the diverticulum of the lagena may occasionally be present and there is never more than one complete semicircular canal. Markedly inhibited grafts of small size consist of an epithelial sac containing at most a single sensory area and are enclosed by cartilage. These types are encountered in grafts of the youngest donors that had been refrigerated for a long time. Two to 5-day otocysts are little affected in their graft development by two to three days of refrigeration, but longer storage progressively inhibits growth and differentiation and so disturb morphogenesis as to make it difficult to distinguish specific parts. A few ears are found in grafts of Group 4 but they are all of the simplest vesicular type.

Sensory areas are usually well differentiated. They vary in number, size, shape and in position within the transplant. Some show innervation whereas others appear to be differentiations of localized areas that do not depend upon innervation for their origin. Their absence possibly corresponds to the failure of appropriate parts of the labyrinth to differentiate. Several grafts of 3- to 4-day otocysts refrigerated four to six days contain striated cupula-like structures over some of the sensory areas associated with blind outpocketings.

Graphic outline drawings were made of all ear grafts. Illustrations of these are not included in this paper since the drawings closely resemble those given in a paper by Waterman and Evans ('40) on the morphogenesis of the avian ear rudiment from embryos of two to five days incubation in chorioallantoic grafts. They emphasize the variability in size, shape, and degree of differentiation in transplants from even the same developmental stage. None of the refrigerated ears attained anyways near the morphological type of development shown by controls of the same total age but histogenesis is more specific and complete. There is no particular increase in the completeness of morphogenesis in the transplants of older stages. A longer graft life does not influence the final result as far as form is concerned, neither does the inclusion of neighboring embryonic tissues with the transplant.

Epidermis and feather rudiments are the most resistant of all ectodermal derivatives and show least change in grafts of any developmental stage and after the longest periods of refrigeration. After long refrigeration that fatally injured most other embryonic chick tissues, typical epidermis still appears in even the oldest grafts along

with cartilage, bone, and even some skeletal muscle. It is found in transplants from embryos refrigerated 22 days. Figure 4 shows developing pigmented feather rudiments in a 7-day graft of skin from a 5-day chick refrigerated 11 days. Feather development is seen to be typical and abundant. Epidermis often lines cyst-like cavities filled with fluid that coagulated in the fixing agents. It may also take the form of epithelial tubes of various sizes, or it may partially outline the general surface of the graft.

b. Mesodermal derivatives. Whole or pieces of mesonephric kidney from the youngest donors did not generally survive even refrigeration for as short a time as two to three days. The very few successful grafts are of small size and have the shape of oval or elongated finger-like masses. Histological differentiation is retarded and disorganized but appears fairly typical as far as it goes. In the sections, the uriniferous tubules are few in number and sometimes widely separated by darkly-stained connective tissue. The amount of intertubular tissue is usually much larger than normal, especially in the periphery of the graft, and it represents probably some host tissue invasion. Regional parts of the tubules are sometimes recognizable, i.e., secreting, collecting, and renal corpuscles. There is no capsule. A bit of Wolffian duct is present at one side of two of the grafts and several collecting tubules open into it. Its wall consists of several layers of muscle cells and the lumen is obliterated in part by large, lightly staining, vacuolated epithelial cells. In one of the smallest grafts only a few short undifferentiated tubules are present. No mesonephric tissue is found in grafts from older embryos or in cases of longer refrigeration, nor recognizable metanephros in any graft.

Cartilage and bone grow and differentiate abundantly in grafts from all embryonic stages and after the longest periods of refrigeration. The cartilage is always typical in appearance (Figures 6, 8, 11), enclosed in a perichondrial sheath, and usually in limb bud grafts is in the form of rounded or elongated masses. No limb-like organization of the cartilage was obtained from any of the limb bud transplants. Replacement of cartilage by perichondrial and endochondrial bone is illustrated by Figures 5 and 14. Figure 5 is a section from a hind limb graft. Membrane bone of typical appearance occurs in a large number of transplants from the head region. Figure 1 is a section from a 7-day graft of a portion of the head region of a 13-day donor refrigerated four days.

Skeletal muscle occurs abundantly in limb bud grafts and those from other regions of the body wall. Like epidermis, cartilage, and bone it shows marked resistance to refrigeration over long periods of time, e.g., 22 days. Figure 1 is a section from an 8-day graft of a 4-day limb bud refrigerated 10 days. Cardiac muscle is found in three grafts from Group 1 (4-day donor), but not in grafts from older embryos or from tissues refrigerated for a longer time. It appears to be the least resistant of the three types of muscle. Figure 15 is a section of a gonad from an 8-day graft of the trunk region of a 4-day embryo refrigerated four days. The transplant appears to be at the indifferent stage of development. Several examples occur in grafts included in Groups 1 and 2 (Table 1). Neither notochord nor spleen were recovered.

c. Endodermal derivatives. Figure 3 is from a 7-day graft of a portion of the trunk from a 9-day donor refrigerated four days and is one of the best grafts recovered. It shows both liver and pancreas. These tissues are the most sensitive to refrigeration of any of the endodermal derivatives tested. Small retarded grafts were obtained from 3- to 5-day embryos refrigerated five to six days (Groups 1 and 2). However, even a 24-hour refrigeration may provoke degenerative changes. As seen in Figure 3, the liver cords are widely separated by connective tissue and spaces containing blood cells but in other grafts the cords lie close together. Cell outlines of the hepatic cells are often quite indefinite. No definite capsule encloses each tissue mass, but they are separated by connective tissue. No trace of bile duct or gall bladder occurs in any graft. Hepatic tissue is generally separated by connective tissue from adjacent intestine within the same graft even in grafts of entire trunk regions of younger embryos. This condition can be attributed to a separation, and subsequent isolation, of the primary rudiments by the activity of connective tissue. In one graft of the trunk region of a 3-day donor refrigerated six days, three separate liver masses are found. Fewer successful grafts are recovered after long periods of refrigeration and in these more extensive degeneration changes had or were taking place.

Pancreatic tissue appears to be about as sensitive as liver to refrigeration. Generally it is found in the same grafts except in those cases where isolated portions of the two organs from older embryos were transplanted separately. Probably it is merely a coincident that

more grafts of pancreas than of liver were recovered from transplants subjected to long refrigeration or from older embryos. In the case of both tissues, transplants of portions of trunk regions of donor embryos are more successful than isolated portions of older organs. Pancreas grafts are retarded in development, small in amount, and the branching cords are often widely separated by connective tissue or small spaces. The cords in Figure 3 are irregular in outline, the cells are darkly stained, and in some cords a slight lumen is visible. No islet tissue was found.

The failure of liver and pancreas tissues of the older embryos to survive after transplantation may be due to other factors than refrigeration alone. For example, Murphy ('16) and Willier ('24) have reported that transplants of adult chicken liver degenerate. According to Sandstrom ('34) chorioallantoic grafts of liver from chick donors up to three days of incubation differentiate normally; whereas in all grafts in which the total age of the tissue is less than 12 days the transplants are histologically normal, but after the twelfth day degeneration becomes apparent. The onset of degenerative processes at this particular time of development is apparently correlated with the biochemistry of the organ as glycogen storage begins in the normal chick about the seventh day of incubation (Dalton). Sandstrom also found that pancreatic rudiments from embryos up to eight days of incubation differentiate normally, zymogen production is suppressed, and the amount of islet tissue is greatly increased above that of the control. The number of islands decreases in grafts from older donors, and grafts from 15- to 19-day embryos show no islet cell. She suggests that the cessation of growth and the subsequent degeneration of liver and pancreas in grafts is due to their inability to function.

Transplants of parts of the digestive and respiratory tracts of older embryos and appropriate levels containing these organs from younger embryos survive long periods of refrigeration. Figure 13 is a photomicrograph of a section of the intestine in an 8-day graft of the trunk of a 4-day embryo refrigerated five days, and Figures 2 and 9 are also of intestine from a 7-day graft of portions of the trunk viscera of a 9-day embryo refrigerated four days. Differentiation is typical but retarded in each case. Intestine, oesophagus, proventriculus, and possibly malformed gizzard are found in grafts of Groups 1, 2, and 3; and intestine and oesophagus in those of Groups 4 and 5. Both of

the latter organs show the appropriate layers of smooth muscle, submucosa, and epithelium. The epithelium of the intestine is one layer in thickness and folded, whereas that of the oesophagus is stratified and nonfolded. All grafts are small in size and have the form of closed oval or elongated vesicles.

Grafts containing structures of the respiratory system show a regional differentiation of the tissues of the transplant into those of the tracheal tube and those of the lung proper. Grafts are found in Groups 1 to 4. Smooth ciliated epithelium lines the tracheal tube and the cartilage in its wall is in the form of irregularly distributed elongated or oval rods. The epithelium may be stratified in places or only a single layer for the most part. In some cases there may be seen several tubes or vesicles lined with this type of epithelium and an elongated tube may show one or more evaginations. Coagulated fluid colored with the cytoplasmic stain is present in some of the vesicles.

Transplanted lung differentiates to a varying extent in grafts from 3- to 6-day donors in the manner described by Rudnick ('33) for fresh tissue providing refrigeration is not too prolonged. Grafts may show folded sacs of respiratory epithelium accompanied by characteristic mesodermal structures, the lung sac may be branched, or an organ-like development may be present. The latter is generally the case in grafts from the older embryos. Differentiation of the respiratory epithelium is typical but retarded for the most part but may show flattening, folding, or even stratification in places. In some cases extrapulmonary and pulmonary portions can be distinguished. The sac type of lung graft is more common in grafts from 3- to 4-day donors and after the longest periods of refrigeration that did not fatally injure the transplant. As Rudnick says, "there is not a point-for-point correspondence between such graft organs and the normal lung; but there can be no doubt that the normal organizing factors are present and that they have produced a comparable structure." Short periods of refrigeration do not apparently interfere markedly with these "organizing factors", whereas long refrigeration brings about disorganization and deformation. On the whole, this conclusion applies for most, if not all, of the transplanted tissues included in this paper.

As stated above, the donor embryos were separated from extra-embryonic membranes and appendages before refrigeration. How-

ever as a matter of curiosity, in two cases whole eggs containing 4- and 7-day embryos were transferred directly from incubator to refrigerator. Representative tissues were transplanted after three and five days of refrigeration. Since no appreciable difference could be detected between the resulting grafts and those of tissues from isolated embryos, it is concluded that this method of storage is practical at least for short periods of refrigeration.

d. Refrigeration of 4- to 9-day embryos for 10 to 12 days and longer. Grafts of different portions of organs of these stages contain chiefly those tissues that are more resistant to storage at low temperatures. Necrotic areas resulting from advanced degeneration of the less resistant tissues are generally absent for the most part showing that prolonged refrigeration had destroyed any recovery capacity. There is present only sharply defined developing tissues including stratified epidermis, hyaline cartilage, intestine, clumps of densely pigmented cells, disorganized eye grafts with folded pigmented tapetum nigrum, folded retina and disorganized lens, lens alone, ganglia, abundant skeletal muscle, numerous feather follicles, cartilage bone, tracheal vesicles, bits of lung, vesicular inner ear enclosed with cartilage, small masses of membrane bone, and heavily stained unrecognizable aggregates, tubes or vesicles of epithelial-like cells. Other nervous tissues, cardiac muscle, liver, pancreas, notochord, and kidney are not found. Essentially similar types of surviving tissues are found in grafts of 3- to 5-day donors refrigerated 15 to 17 days. Increase in length of refrigeration produces further disorganization of morphogenesis in organ transplants particularly noticeable in eye and internal ear, progressively fewer and smaller grafts are recovered, the amount of unidentifiable epithelial-like tissue increases, and differentiation is retarded. After 21 to 22 days of refrigeration only epidermis, pigmented feather follicles, cartilage, bone, some skeletal muscle, and epithelial aggregates are found in the few grafts recovered.

Discussion

Certain warm-blooded adult animals will withstand low temperatures (hibernation) because adaptive properties have been developed, and chilling has long been employed as an anaesthetic in laboratory experimentation (Parker, '39). However, in the hibernating adult the histologic and gross changes in tissues and organs are associated

with the ability to live through unfavorable weather conditions (Holzapfel, '37). On the other hand, any considerable variation in temperature is not a natural occurrence in the environment of warm-blooded embryos. In the case of the chick retarded and abnormal development and even death of the individual usually results when the developmental temperature varies but a few degrees either above or below the norm for incubation. The general opinion appears to be that tolerance to temperature change is only gradually acquired after hatching or birth.

Evidence is gradually accumulating to show that embryonic tissues of both cold- and warm-blooded vertebrates may be refrigerated at low temperatures, for a time at least, that kill the individual and the stored tissue may be later transplanted successfully to suitable culture media where recovery, growth, and differentiation will take place; and that different embryonic tissues vary in their sensitivity to refrigeration. This variation may indicate an explanation of the process of death of the embryo cultivated at subnormal temperatures (Needham, '42). Recovery ability is conditioned not only by the variable sensitivity of tissues, but also by the temperature (above freezing) and for the most part by the length of exposure and hence the susceptibility to anoxia. A suitable temperature for preserving embryonic vertebrate cells alive for any length of time appears to be in the neighborhood of 5° C. (Bucciante, '31; Luyet and Gehenio, '40; Stone, '43; Waterman, '39; this paper).

Bucciante (loc. cit.) has shown by culture in vitro test that tissues of 6- to 12-day chick embryos vary in survival time when kept in Ringer's solution at 5° and 10° C., over 21 days for skin to three days for the blood elements in spleen and for hepatic cells. Cooling down to 0° C. lengthened the survival time somewhat but temperatures below this shortened the time. Ringer's solution appeared to wash out substances necessary for growth. No tissues survived —30° C. He also took bits of chick embryos and left them under sterile conditions at room temperature. Skeletal muscle grew in tissue culture after 20 days, cornea after 18 days, skin after 14 days, heart muscle after 6 days, and liver after 2 days. According to Shipp ('37) embryo juice may be obtained from 8-day chick embryos which have been stored at 10° F. for over six months, and a greater growth is secured than from fresh embryos. There is general agreement that

the susceptibility of the chick embryo to periods of chilling increases steadily and continuously throughout development (Needham, '42) and the embryos of birds are more resistant to low temperatures than the adults, but little seems to be known of the time at which the resistance drops (Luyet and Gehenio, loc. cit). Processes continue within stored eggs that lead eventually to autolysis (Swenson, '38) and partially incubated eggs are less resistant than fresh eggs. In general the sperm and eggs of animals are more resistant than growing differentiating tissues of the same species. According to Edwards (quoted by Luyet and Gehenio) chick embryos respond like cold-blooded animals to temperature change during the first 20 days of incubation.

Hetherington and Craig ('39) exposed whole 11-day chick embryos and isolated hearts or pieces to low temperatures and explantations were made at subsequent intervals until no further growth was obtained in the cultures. They found that small fragments of chick heart may be stored in Ringer-Tyrode solution at 0° C. for as long as 15 days and at 20° C. for 11 days with little apparent effect upon viability in tissue culture. Sub-zero temperatures rapidly kill cells as do supra-normal temperatures of 50° and 52° C. Survival of heart muscle and fibroblasts is dependent upon time-temperature and mass factors. Frozen-dried chick embryo juice is active after three months of storage. There occurs a latent period of as much as three days duration before growth is visible in the cultures especially after treatment with sub-zero temperatures. Of several possible explanations of this, they favor the idea of a recovery period taking place in the cells. If the accumulation of toxic products in the whole embryo enters into the picture, they found no experimental evidence. In the case of reduced size of the stored material, the availability of oxygen and perhaps secondarily the presence of glucose in the storage medium are important factors in the longer resistance of the cells. At 0° C. fibroblasts and heart muscle in the whole embryos survived 291-312 hours, in heart alone 360 hours, and in heart fragments 384 hours. The cells do not die off in slow progression, but rather die en masse within definite time limits.

The results of the present study, in which whole chick embryos of 3 to 16 days of incubation were stored between layers of moistened cotton at 0°, 5°, and 10° C. and chorioallantoic transplantations were

employed as a test of viability, are comparable to those described by Bucciante (loc. cit.). Variability between the survival times of various tissues is not due to drying or to the loss of materials through solution, and this technique of storage furnished no source for any of the materials necessary for life as might be present in a culture medium. Certain tissues were found more sensitive to storage and low temperature than others. For instance, nervous tissue such as brain and spinal cord, heart, gonad, kidney, and spleen are affected by even two days of refrigeration; spinal cord is more resistant than brain tissue, kidney, and spleen; notochord and metanephros were never recovered; and gut, trachea, lung, eye, inner ear, and ganglia survive many days. More specifically, in grafts from 4- to 9-day embryos refrigerated for 10 to 12 days there occur skin, cartilage, intestine, dense clumps of pigmented cells, eye tissues, lens, bone, muscle, disorganized spinal cord, young feather follicles, trachea, lung, ganglia, ear; but no kidney, heart, pancreas, liver, brain, or notochord. This list includes derivatives from each germ layer. With the exception of spinal cord and ganglia, practically the same types of tissues appear in grafts of 14-day donors refrigerated for 9 days and of 3- to 5-day embryos refrigerated for 10 to 12 days. Cartilage, bone, skin, feather rudiments, and skeletal muscle recover to some extent even after three weeks of storage.

To what extent are the survival values of refrigerated chick embryonic tissues comparable to those of mammals? Unfortunately, observations have been reported for only a few mammals, stages have been limited and different methods have been employed for testing. Whole blastocyst and primitive streak to early somite stages of the rabbit resume development in vitro after refrigeration of the pregnant uterus for 30 hours. Tissues of 13- and 15-day embryos have been tested for viability after refrigeration of the whole embryo by transplantation to the omental bursa of the adult of the same species (Waterman, '39). With the exception of notochord and brain, refrigeration for 1-2 days has no appreciable effect. After 4-5 days of refrigeration pancreas, liver and kidney seldom resume development. Refrigeration produces retardation, but otherwise little change except in morphogenesis as shown by increased disorganization of organ grafts. Grafts of 8-day refrigerated donors contain epidermis, hair, cartilage, skeletal muscle, bone, lens, trachea, disorganized eye, in-

testine, lung, vesicular inner ear, etc. After longer periods of storage the chief tissues to show successful recovery are epidermis, hair, cartilage, and bone.

Simonin ('31) subjected fragments of embryonic tissues of mouse, rat, and ox to temperatures from 0° to —15° C. He obtained some growth in culturing the tissues after they had been at 0° for 20 days or at —5° for five days, providing no ice had been formed in them; —15° was always lethal in a short time. Nerve cells and liver were most sensitive; whereas heart, lung, and intestine showed greater resistance. Pincus and Shapiro ('40) and Shapiro ('41) have reported parthenogenetic activation of unfertilized rabbit eggs by means of cold temperatures. Nicholas has found that rat embryos withstand six hours at 37.5° C. without histolysis and 12 hours at 24° C., after which histolytic changes become visible. After 72 hours at 24° C. the embryo maintains apparently normal form but cellular structure is atypical and degenerative changes are visible. No differentiation occurred during the experimental period. When the pregnant uterus is placed in a subcutaneous position, development is normal. Nicholas ('38) has also studied the development of rat embryos in a circulating medium. Temperature variations within rather wide limits, e.g., 70° to 110° F., seemed to have slight effect upon comparable stages, whereas temperatures below 80° F. affected the heart beat. Numerous studies of tissue and organ survival after death have been made of adult mammals and these show that tolerance to anoxia decreases after birth (Alvarez, '37; Needham, '42; Payne and Meyer, '42; Glass, Snyder, and Webster, '44) and that certain tissues and organs survive exposure to low temperatures and may be stored for surgical use.

The studies briefly reviewed above demonstrate that bird and mammal embryonic tissues are comparably sensitive to refrigeration and storage in spite of the difference in temperature at which normal development takes place. Any marked difference in the length of the survival time at similar temperature is probably not inherent in the tissues themselves, but may in part at least be due to the difference between environments used to test survival. The chorioallantoic membrane of the chick is an embryonic organ, whereas the greater omentum of the adult rabbit possesses an abundance of phagocytic cells and exhibits sudden and intense reactions towards foreign bodies.

Both chick and mammal embryonic tissues appear to respond in a general way like those of cold-blooded animals to refrigeration during early development (cf. Edwards, loc. cit.). It is still a question as to what part the state of embryonic differentiation and the onset of function play in embryonic tissue survival. It would appear from a survey of the available literature that younger embryonic tissues of warm-blooded animals are more resistant to extreme oxygen deprivation during refrigeration than those from older embryos in which structural organization is more highly differentiated, and that this capacity to resist anoxia continues to decrease after birth or hatching (Needham, '42; Glass, Snyder, and Webster, '44).

Various methods of testing tissue survival after death of an animal have been employed, e.g., physiologic, histologic, culture in vitro, and transplantation. Alvarez (loc. cit.) cites records from the literature to show that in adult mammals endings in the central part of the nervous system, and perhaps some ganglion cells, cease functioning within a minute or two after circulation stops. Susceptibility to anoxia decreases from cortex through the medulla and to the spinal cord. Consciousness and most reflexes disappear immediately. Nerve fibers and motor nerve endings will function for an hour or two when left in the uncooled animal, whereas cooling and the presence of blood in the tissues will preserve the function time a little longer. Histologic changes likewise begin to occur soon after death; 3½ hours for ganglion cells in exsanguinated gut, 10 minutes for those in the brain, 30 minutes for spinal ganglion cells, and a little longer for cells in the medulla and in the anterior horns of the spinal cord. Yet well-stained preparations of ganglion cells from the intestine have been made 10 to 15 hours after death and seven or eight days if cooled. Alvarez was unable to determine from histologic study whether or not ganglion cells are capable of function. The development of vacuoles and granules having an affinity for neutral red has been used as a sign of dying. When the tissue dies, the color leaves these structures and diffuses throughout the cell. The granules and vacuoles may form before the tissue can be removed from the dead animal (brain, intestinal epithelium, heart, skeletal muscle), around four days after death (liver, pancreas), or even 10 days (smooth muscle of bladder and uterus).

Histologic methods are not as delicate a test as either culture in

vitro or transplantation. In the present study it was found that changes in the physical appearance of cells, e.g., slight shrinkage, pycnotic nuclei, formation of minute vacuoles, appearance of spaces in tissues, are only indicative of approaching death and do not discriminate between survival times and recovery ability of different types of tissues. After three weeks of refrigeration cartilage, bone, skeletal muscle, and epidermis showed striking physical change at the time of transplantation yet they recovered after transplantation. The differential survival of various nervous tissues demonstrated by the present study parallels that reported by Alvarez above. Finally it would seem from the reports of culture in vitro tests that this method is even a more delicate one than transplantation of embryonic tissues.

Death of refrigerated tissues, in which the time factor is important, has been attributed (cf. Luyet and Gehenio, loc. cit.) to lack of oxygen, disturbance of physiological functions, chemical changes, accumulation of toxic products, alteration of permeability, dehydration, changes in viscosity and adsorptive properties, and to processes of solidification, precipitation, and coagulation. While dehydration might be looked upon as a contributory factor in the present study because of the method of storage, Alvarez (loc. cit.) reports from the literature studies in which bits of chick embryo, heart muscle, and blood vessel wall were kept alive in a desiccated condition for periods as long as six months. As mentioned above, Hetherington and Craig found no experimental evidence in support of the view that the accumulation of toxic products conditions the length of tissue survival of stored whole chick embryos. On the other hand it is well known that the adult nervous system has a high metabolic rate, is the first organ system to suffer when the heart stops beating and certain parts of the system are more susceptible than others to anoxia (see also above). MacArthur and Jones ('17) have shown that in the adult central nervous system the metabolic rate of the tissues decreases from the gray matter of the cerebrum to the white tract at the lower end of the spinal cord. According to Romanoff ('43) the rate of respiration of various regions and tissues of the chick embryo changes with development and with the relative dominance of tissues of high or low metabolic activity in these regions. Kidney shows the highest rate; next in order are brain, gonads, lung, muscle, and liver. The rate of oxygen consumption declines with increasing incubation age

in the case of liver, lungs, heart, leg muscle, and gonads. In adult animals the kidney and retina of the eye show the highest respiratory rate; next in order are gonad, nerve tissue, liver, heart, spleen, lungs, and lastly resting muscle. On the basis of the above information, the differences in metabolic rate and sensitivity to anoxia of chick embryonic tissues may contribute more than any other factors to an explanation of the results obtained in the present study. From this it may be inferred that differential tissue sensitivity to refrigeration and storage appears very early during development.

Summary

1. A comparative study by means of chorioallantoic transplantation has been made of the viability of embryonic chick tissues and organ rudiments of different developmental ages following refrigeration and storage of whole embryos at temperatures of 0°, 5°, and 10° C. for periods of time ranging from one to 22 days. Embryos of four to 16 days incubation served as donors.

2. The most suitable temperature for preserving embryonic cells alive for any length of time appears to be in the neighborhood of 5° C. Different tissues vary in sensitivity to refrigeration, and differences in metabolic rate and resistance to anoxia may contribute more than any other factors to an explanation of the results obtained in this study. Nervous tissues survive only a few days of refrigeration; spinal cord is more resistant than brain tissue; heart, gonad, kidney, and spleen are affected by even two days of refrigeration; notochord and metanephros were not recovered; gut, trachea, lung, eye, inner ear, and ganglia survive many days; and cartilage, bone, skin, feather rudiments, and skeletal muscle survive even three weeks of storage. Death occurs quickly during storage at 0° C. The appearance of refrigerated tissues and differentiation within the grafts are described.

3. Results obtained with chick embryonic tissues are comparable to those reported previously for the rabbit. Any difference in survival times is probably due to the test environment, e.g., chorioallantoic membrane vis. the adult omental bursa. There seems no reason to believe that the higher incubation temperature required by the chick for normal development renders its tissues more susceptible than those of the rabbit to refrigeration at the same temperatures. Histological study of the donor tissues gave no precise indication of the

recovery capacity of these tissues after transplantation. The present study contributes evidence to the general conclusion that many embryonic chick tissues and organ rudiments successfully survive refrigeration and storage for several days at least, and that survival is conditioned by temperature and length of refrigeration. Morphogenesis of organ transplants, i.e., internal ear, spinal cord, eye, is progressively more disturbed and developmental retardation increases as a result of prolonged refrigeration.

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THE PRENATAL GROWTH OF THE CAT: XV. THE WEIGHT OF THE MUSCULATURE IN THE FETAL AND IN THE ADULT CAT

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The weight of the skeletal muscle, its rate of increase, and the amount of skeletal muscle gained per unit of food is obviously an important consideration in domestic animals raised for food. However, there are very few studies giving these data. Loeffel has studied the weights of the musculature in the pig of commercial slaughter weights but has not yet published his excellent data. Latimer (1924) and Mitchell, Card, and Hamilton (1926) have reported on the musculature of the chicken, and Latimer and Rosenbaum (1926) for the turkey hen.

The weight of the human musculature has been studied by Hasselwander (1931), Scammon (1923 and 1933), and Wilmer (1940). Two of the many reports on the albino rat are by Jackson and Lowrey (1912), and Phillips and Gilder (1940). Scammon (1923) says, "We know comparatively little regarding the postnatal development of the muscular system." This is equally true for the prenatal growth.

So far as is known there are but two reports on the weights of the skeletal muscles in the cat. Sedlmair (1899) gives the weight of the musculature in one normal adult cat and the average of four specimens is reported by Welcker and Brandt (1903).

MATERIALS AND METHODS

The weights of the musculature described in this paper are from 229 fetal, 35 newborn, and 104 adult cats. These have been described in the earlier numbers of this series and will not be repeated here. After the removal of the skin, the viscera, all easily removable fat, mesenteries and large vessels, the remaining musculature, skeleton, and central nervous system were weighed. Then the muscles were carefully removed. Several methods were employed but the most satisfactory was by cutting them off with a pair of scissors. It was

found that this was the most rapid method and also gave the cleanest skeleton. Then the skeleton and the enclosed nervous system were weighed and the difference gave the weight of the skeletal muscles. All of the parts of the cat not being worked on were kept in a moist chamber to prevent drying. The head and the four limbs had been removed from the body, in the preparation of one of the earlier studies. It was felt that this method gave more accurate weights of the muscles than a direct weighing of them. For a further discussion of the methods see the preceding paper of this series (Latimer, 1944). This method gives more than the weight of just the muscular tissue, for included are the tendons, the blood vessels and nerves within and between the muscles and a variable amount of fat. The largest variable factor is probably the amount of fat included with the muscles. The ligaments were left and weighed with the ligamentous skeleton.

The muscles of the fetal and newborn cats were weighed on a chemical balance sensitive to 0.1 milligram and the weights of the musculature of the adults, on a laboratory balance sensitive to 0.1 gram. The fetal and newborn cats had been preserved in formalin but the adults were weighed in the fresh condition. In all other respects the fetal, newborn, and adult cats were treated in the same manner, and the dissections and weighings were made by the same person.

The formula for the growth of the musculature of the smaller fetal cats plotted on body length was developed by trial and error in the same manner as the earlier curvilinear formulae in this series (Latimer, 1933). All of the other curves are straight lines and the empirical formulae for them were developed by the method of least squares (Snedecor, 1938). The formulae used for the determination of the statistical constants for the adult cats are those generally so used and are given by Dunn (1929). All of the computations were carefully checked, and carried to more decimal places than given in the tables.

Much of the routine statistical work on the adult cat was done by Mr. Dale L. Lemon and I wish to take this occasion to express my most hearty appreciation of his careful, accurate work.

Fetal Musculature

The growth of the muscles of the fetal and newborn cats is shown

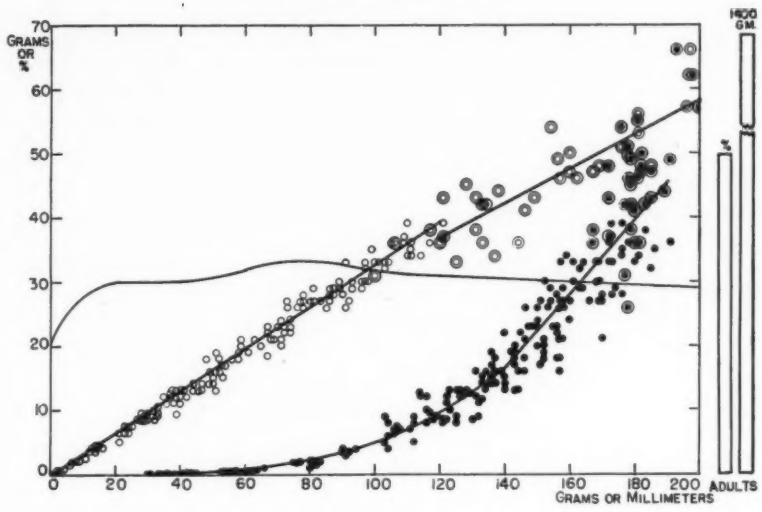

FIGURE 1

The curve starting at 20 per cent and without any cases represents the weight of the musculature as a percentage of the body weight. The curve starting at 0.3 grams and with the weights of the musculature of each specimen shown as a circle for the fetal cats and as double circles for the newborn cats represents the growth of the musculature plotted on body weight. The curve concave superiorly at first and with the weights of the musculature of the individual cats shown as dots for the fetal cats and as dots enclosed by circles for the newborns, shows the growth of the muscles plotted on body length. Empirical formulae for these curves are given in the text. The shorter column in right margin gives the average percentage weight of the muscles in the adult males. The taller broken column, the absolute weight of the average male musculature. The ordinates give either weight in grams or percentages and the abscissae either body weight in grams or body length in millimeters.

in Figure 1. The straight line starting at 0.3 grams and with the weights of the muscles of the individual fetal cats shown as circles gives the growth in weight of the musculature plotted on body weight. The second curve starting at 121 grams of body weight and with the weights of the individual newborn cats shown as double circles, represents the increase in weight of the muscles of the newborn cats and its rate of increase is less rapid than that for the fetal cats as shown by the smaller angle with the line of abscissae. The empirical formulae for these two curves are:

$$Y = 0.328\ X - 0.06,$$

from 0.3 to 120 grams of body weight, and for the newborn cats, or from 121 to 200 grams of body weight,

$$Y = 0.269\ X + 431.$$

In both of these formulae Y represents the weight of the muscles in grams and X, the body weight also in grams. The average percentage deviation of the calculated from the observed averages for each ten grams increase in body weight is 5.56 per cent for the first curve and 4.10 per cent for the second curve.

The lower curve, beginning at 30 millimeters of body length and with the weights of the musculature of the fetal cats shown as dots and that of the newborn cats as dots enclosed by circles, was drawn from the following empirical formulae. From 30 to 140 millimeters of body length,

$$Y = \frac{(0.1\ X)^{3.7}}{1052}$$

and from 140 to 190 millimeters of body length,

$$Y = 0.59\ X - 66.434.$$

In both of these formulae Y represents the weight of the muscles in grams and X, the body length measured in millimeters. The second formula for the rectilinear part of the curve gives the weight of the muscles of the larger fetuses and for the newborn cats as well. The average deviation of the calculated from the observed averages for the first part of this curve is 10.70 per cent and for the second or rectilinear part of the curve the average deviation is but 1.89 per cent. This average percentage deviation of over ten per cent is about as large as one should have. The weight of the musculature, as well as some of the other parts of these cats, is more accurately expressed in terms of body weight than in terms of body length.

The lighter line without any cases, represents the weight of the muscles expressed as a percentage of the body weight and plotted on body weight. The musculature starts at about 20 per cent of the body weight, rises rapidly at first and then more slowly to its maximum of about 33 per cent at about 80 grams of body weight and then slowly and uniformly decreases to a little less than 30 per cent. The shorter column at the right represents the average percentage weight of the musculature in the adult male cat as 49.79 per cent. The percentage is slightly greater in the female as shown in Table 1. The

taller broken column represents the average weight in grams of the adult male. Further statistical details concerning the adult cats are given in Table 1.

TABLE 1

WEIGHTS, PERCENTAGE WEIGHTS, AND SIGNIFICANT DIFFERENCES OF THE MUSCULATURE AND THE LOCOMOTOR SYSTEM IN THE ADULT CATS

	Males		Females		
	Average weight	Coefficient of variation	Average weight	Coefficient of variation	Significant difference
Muscles in grams	1400.23±37.30	27.93±2.03	1247.38±29.09	23.95±1.74	3.23
Locomotor system (grams)	1792.80±41.85	24.95±1.75	1540.26±31.14	21.62±1.46	4.84
Muscles (percentage of body weight)	49.79± 0.33	7.02±0.48	49.86± 0.39	8.11±0.56	0.13
Locomotor system (percentage of body weight)	63.59± 0.26	4.44±0.29	62.94± 0.31	5.21±0.35	1.61

The curves in Figure 1 seem to indicate that during the fetal period the musculature increases as an arithmetical function of the body weight, with a slightly slower rate of increase in the newborn, and as a geometrical function of the body length in the smaller fetuses, and then in the later part of the fetal period and in the newborn, as an arithmetical function of the body length. The muscles increase to their maximum percentage of the body weight some time before birth and during the later part of the fetal period there seems to be a slight decrease to the percentage value at birth. The average of all of the newborn is 29.80 per cent. Both Hasselwander (1931) and Wilmer (1940) give slightly greater percentage weights for the human muscles at birth than for the sixth or seventh fetal month. They give no other percentage weights for the fetal period and so it is hard to compare the growth of the musculature of the cat and man in the fetal period. Scammon (1933) says that the human musculature "grows rather slowly in the young embryo but increases to about 25 per cent of the body at birth."

The growth of the cat skeleton during the fetal period has been reported in the preceding paper of this series (Latimer, 1944). The weights of the skeleton and the musculature were combined for each specimen and the combined weights, or the weights of the locomotor

system, were plotted as has been done for the musculature. The resulting curves are not given for they closely resemble the curves for the musculature. The empirical formulae for the weights of the locomotor system are given for determination of the weights of the combined skeletal and muscular system from either body weight or body length. From 3 to 120 grams of body weight the formula for the weight of the locomotor system is,

$$Y = 0.45\ X - 0.482, \text{ and from 121 to 200 grams,}$$
$$Y = 0.365\ X + 7.827.$$

In the above formulae, Y represents the weight of the locomotor system in grams and X, the body weight also measured in grams. The average percentage deviation of the calculated from the observed values is 3.37 per cent for the first curve and 3.61 per cent for the second curve.

The increase in weight of the locomotor system plotted on body length is given in the two following empirical formulae. From 30 to 140 millimeters of body length,

$$Y = \frac{(0.1\ X)^{3.7}}{750} + 0.068, \text{ and from 140 to 190 mm.,}$$
$$Y = 0.82\ X - 92.503.$$

In the above formulae, Y represents the weight of the musculature in grams and X, the body length measured in millimeters. The average percentage deviations for these formulae are, 4.82 for the first formula and 1.03 per cent for the second formula, or from 140 to 190 millimeters.

The percentage curve is similar to that shown in Figure 1 for the musculature except that the maximum of 45 per cent comes at about 90 grams instead of at 80 as is shown for the muscles. The average for all of the newborns is 41.5 per cent. This percentage curve resembles that of the muscles although it is modified somewhat by the weight of the skeleton. The initial drop in the percentage curve for the skeleton (previous paper of this series) decreases the initial rise shown in the muscles and the peak at the maximum of the curve for the muscles is flattened out somewhat and comes a little later as has been stated. The muscles form so much larger a proportion of the body weight that they would tend to overshadow the percentages of the skeleton.

Adult Cats

The average weight in grams and the average percentage weight of the musculature and the locomotor system, the coefficients of variation and the significant differences, or the differences divided by the probable error of these differences, for the adult male and female cats are given in Table 1. The weights in grams of the musculature and the locomotor system are significantly greater in the male cats. The locomotor system likewise forms a slightly but not significantly greater percentage of the body weight in the males, but the muscles form a larger, but again not significantly larger, percentage of the body weight in the females. It is interesting to see that both weights in grams are less variable in the females but the percentages of these two systems are less variable in the males. This greater uniformity in the male percentage weights may be explained by the better correlations between the weight of the musculature and the body weight, body length and the weights of many of the organs as shown in Table 2. The coefficients of variation are greater in the males by 16.6 per cent for the muscles and 15.4 per cent for the locomotor system when the absolute weights are used and the coefficients for the per-

TABLE 2

Correlations with the Weight of the Musculature

	Males	Females
Body weight	+0.978±0.004	+0.917±0.015
Body length	+0.777±0.038	+0.520±0.071
Integument	+0.913±0.016	+0.894±0.020
Heart	+0.908±0.017	+0.754±0.042
Spleen	+0.609±0.060	+0.609±0.061
Brain	+0.248±0.090	+0.279±0.090
Spinal cord	+0.527±0.069	+0.416±0.081
Eyeballs	+0.673±0.052	+0.384±0.083
Digestive tube weight	+0.641±0.056	+0.403±0.082
Digestive tube length	+0.340±0.084	+0.392±0.082
Liver	+0.607±0.060	+0.521±0.071
Pancreas	+0.557±0.066	+0.555±0.067
Lungs (clear lungs only)	+0.611±0.070	+0.520±0.086
Kidneys	+0.844±0.027	+0.964±0.007
Bladder, urinary	+0.870±0.023	+0.405±0.081
Gonads	+0.879±0.022	+0.727±0.046
Uterus	———	+0.436±0.083
Thyroid	+0.690±0.050	+0.349±0.086
Suprarenals	+0.613±0.060	+0.501±0.073
Hypophysis	+0.170±0.094	+0.411±0.082

centages are greater in the females: 15.5 per cent for the muscles and 17.6 per cent for the locomotor system.

The average percentage weight of the musculature for the four cats given by Welcker and Brandt is 44.92 per cent or less than the average for this group but well within the range of the percentages of this group. The percentage weight of the muscles for the one control specimen is given by Sedlmair as 57.2 per cent. This is much greater than my averages and there was only one male cat with as high a percentage and the highest percentage in the females was 56.1 per cent.

If we compare the percentage weight of the musculature of these cats with the percentage of other forms which are known, we find that the relative weight of the muscles of the cat is surpassed only by the turkey (58.5). The domestic fowl ranks just slightly below the cat with a percentage of 48.1, followed by the albino rat, 45.4 (Jackson and Lowrey) or 45.49 (Phillips and Gilder); man, 40-45 (Scammon, 1933) or 43.07 (Wilmer) and the much lower values of 33-36 per cent given by Hasselwander (1931). The muscles of the pig form the smallest percentage, or from 37 to 22 per cent (Loeffel). He found the largest specimens with the smallest percentage weight of the muscles, due largely to the increase in the amount of fat in the heavier pigs. This small percentage is further explained by the fact that some small muscles of the head and the feet were not included.

The body weight of the cat increases 195 times during the fetal period and the muscles increase 380 times during the same period or almost twice as much as the body. In the postnatal period the musculature increases just a little more than the total body increase. The increase for the total body weight for the males is 22.3, and for the females 19.9 times (Latimer, 1936) and for the muscles the increases are respectively, 25.4 and 22.6 times. These are total increases for the entire period. So far as is known there are no data on the rates of postnatal growth of the musculature of the cat so we have no idea in what part of the postnatal growth period these increases take place. The postnatal growth of the muscles in man are described by Scammon (1923). He says (p. 295), "Apparently the muscles do not grow as fast as the remainder of the body during infancy but

in later childhood they increase so rapidly that at puberty, as in later life, they form 40 to 45 per cent of the total body weight."

The data in Table 1 are averages and they will predict the weights and percentage weights for average cats but to give a more accurate method of estimating the weights of the muscles and the locomotor system in all adult cats, empirical formulae are given as has been done for the other organs and systems. The following empirical formulae are valid for the males from 1700 to 4500 grams of body weight and from 450 to 600 millimeters of body length. For the females, the formulae are valid from 1500 to 3500 grams of body weight and from 450 to 570 millimeters of body length.

The weight of the musculature in the adult male cats may be estimated from the following formula, based on body weight,

$$Y = 0.529\ X - 86.04.$$

The locomotor system may be computed from the following:

$$Y = 0.617\ X + 52.09.$$

In the above formulae Y represents the weight of the musculature or locomotor system in grams and X, the body weight also in grams. The average percentage deviation of the calculated from the observed values is 1.39 for the musculature and 0.76 for the locomotor system.

The same adult male systems plotted on body length have the following formulae. For the musculature on body length,

$$Y = 8.5\ X - 3001,$$

and for the weight of the locomotor system plotted on body length,

$$Y = 10.11\ X - 3450.65.$$

Again Y represents the weights of the systems in grams and X now represents the body, or nose-anus, length measured in millimeters. The average percentage deviations are, 3.86 per cent for the muscles and 4.10 per cent for the locomotor system. Again these two sets of formulae show that the body weight provides better abscissae than the body length, for the first set of formulae, based on body weight are better fitted than these last two.

For the adult female musculature plotted on body weight,

$$Y = 0.522\ X - 32.29,$$

and for the locomotor system,

$$Y = 0.616\ X + 29.87.$$

Y represents the weight of the systems in grams and X, the body

weight also in grams. The average deviations for these formulae are, 2.70 per cent for the muscles and 2.07 for the locomotor system.

The same systems of the adult female cats plotted on nose-anus, or body length are given by the following formulae. For the musculature,

$$Y = 6.34\ X - 1995.02,$$

and for the locomotor system,

$$Y = 7.94\ X - 2552.46.$$

Again Y represents the weight of the systems in grams and now X represents the body length measured in millimeters. The average percentage deviations are 5.80 for the muscles and 4.57 for the locomotor system. These deviations are larger than the deviations for these systems plotted on body weight.

The percentage weights of the muscles and of the locomotor system were plotted on body weight and curves drawn in the same way as for the absolute weights. These curves are not shown. The percentage weight of the muscles in the adult males started at 46 per cent, increased regularly to 52 per cent at 3300 grams of body weight and then decreased to 50 per cent at 4500 grams of body weight. The male locomotor system remained at 64 per cent of the body weight from the smallest adult cats up to a body weight of 3300 grams and then it decreased regularly to 61 per cent at 4500 grams of body weight.

In the adult female cats, the musculature formed 48 per cent of the body weight in the smallest cats, reached its maximum of 52 per cent at 2700 grams of body weight and then decreased to 49 per cent at 3500 grams of body weight. This curve very closely resembles that for the male muscles. The curve for the percentage weights of the female locomotor system, unlike that for the males, rises uniformly from 59 per cent at 1500 grams of body weight to its maximum of 64 per cent at 2700 grams and then, like that for the males, it decreases uniformly to 60 per cent at 3500 grams. In both sexes, both systems decrease in percentage weight in the heavier cats. This without doubt is due to the increase in the amount of fat in these larger cats. Loeffel in his work on the development of the muscles in pigs from 150 to 400 pounds of body weight shows that the muscles decrease in percentage weight and the amount of fat increases through-

out this period. The muscles in these heavier cats are still increasing in absolute weight although decreasing slightly in relative weight due to the more rapid increase of fat in the larger specimens. The variation in the initial parts of the curves for the locomotor systems of the two sexes is interesting. When the two curves for the absolute growth are examined the curve for the females seems to rise a little more rapidly at first than that for the males. The difference does not seem to be enough to account for the entire difference between the two percentage curves. Another explanation is that some of the other systems or organs develop more precociously in the females and then the locomotor system develops later on.

Ratio of Musculature to Skeletal Weight

The ratio of the musculature to the weight of the ligamentous skeleton was determined for each of the fetal, the newborn and the adult cats. The ratio for each cat was plotted and smoothed curves drawn through the cases as was done for the percentage weights and these smoothed curves without the individual cases are shown in

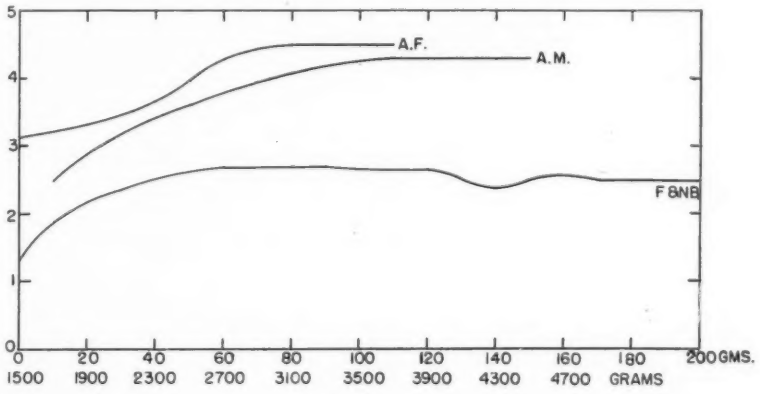

FIGURE 2

Smoothed curves showing the ratios of the weight of the muscles divided by the skeletal weight. The upper curve shows the changes in the ratios for the adult female (A.F.) cats. The middle curve shows the ratios for the adult males (A.M.) and the lowest curve the ratios for the fetal and newborn cats (F. & N.B.). The upper line of abscissae, or from 0 to 200 grams is for the curve for the fetal and newborn cats and the lower line, or from 1500 to 4700 grams for the adults. The ratios are given at the left.

Figure 2. In the smallest fetal cats the muscles were 1.3 times the weight of the skeleton. This increased to its maximum of 2.66 from 60 to 90 grams of body weight and then decreased slightly to birth as shown in the lower line, marked F. & N.B., in Figure 2. Then in the early newborn or from 120 to 160 grams of body weight there is a drop to 2.40. This is very likely due to an early postnatal decrease in the rate of growth of the musculature as shown in the curve for the weight of the muscles plotted on body weight in Figure 1. The similar curve for the skeleton shown in No. XIV of this series shows no decrease in its postnatal growth. The ratios remain constant for the larger newborn cats, or from 170 to 200 grams of body weight.

The two upper curves represent the changes in the same ratio for the adult cats. The middle curve, marked A.M., shows the ratios for the adult males. In the males the musculature is 2.5 times the weight of the skeleton in the smallest adult cats and this increases to 4.3 times the skeletal weight in the heavier adult males. The muscles are about 3.1 times the skeletal weight in the smallest females and this ratio increases rather irregularly as shown in Figure 2, to a maximum of 4.5 times the skeletal weight in the heaviest adult females. In other words the musculature in the smallest fetal cats is very slightly heavier than the skeleton. This increases in the fetal period, with a slight postnatal decrease and then is slightly heavier in the smallest adults and increases to its maximum in the heaviest adults of both sexes. Doubtless some of this increase in the adults is due to the accumulation of fat within and between the muscles; fat which could not be removed in gross dissection.

These ratios in the adult cats were studied statistically, and the averages are 3.85 ± 0.078 for the females and 3.71 ± 0.076 for the males. The musculature is slightly heavier compared to the skeleton in the females but this is not significant as the significant difference is but 1.29. There is a good deal of variation in these ratios for the coefficient of variation for the females is 20.83 ± 1.49 and for the males, 21.35 ± 1.50.

Correlations

The correlations of the weight of the musculature in the adult males and in the adult female cats, with body weight, body length and with the weight of many of the organs is shown in Table 2. All of the correlations are positive, although some are too low to be sig-

nificant. The average of the 19 correlations with the male musculature is +0.656 and the average of the 20 female correlations is +0.548, or the average of the correlations is slightly less than 20 per cent higher in the males. As in many of the other parts of these cats, the muscles of the males are more variable (Table 1) and yet they, and most of the other organs and systems, seem to vary as a unit for the correlations are higher in the males. The correlation of 0.978 for the male body weight and the weight of the muscles is very high, and it should be, when one remembers that the muscles form almost 50 per cent of the total body weight. The correlations of the muscles and the organs are slightly better (by 1.5 per cent) than the correlations of the skeletal weight with the same organs in the males and are better by 22.3 per cent in the females. There are no correlations of 0.9 with the skeleton in either sex.

The highest correlation in the females is not with the body weight but with the kidneys. The next four correlations, in order of decreasing size are: body weight, integument, heart and gonads. These four are the four highest correlations in the males and in the same order. The kidneys fall in the sixth place in the males.

The lowest correlation in both sexes (0.170) is found in the male with the hypophysis and the musculature. The brain is next higher with a correlation of 0.248 in the males and 0.279 in the females. These two (hypophysis and brain) and the length of the digestive tube are the only three correlations which are below 0.5 in the males. These three and six other correlations in the females are below 0.5.

In general the correlations with the muscles are higher on the average in the males but the averages are fairly good in both sexes. There are more low correlations in the females than in the males.

Summary

The muscles and the locomotor system increase as arithmetical functions of the body weight in the fetal and in the newborn cats, but as geometrical functions of the body length in the smaller fetal cats. In the later part of the fetal period and in the newborn cats each system increases as an arithmetical function of the body length.

Empirical formulae are given for the determination of the weight of either system from either the body weight or the body length in the fetal and in the newborn cats. The usual statistical constants

are given for both systems in the adults and, in addition, regression formulae have been developed for both systems for either body weight or body length for the males and females separately. All of the relationships in the adults are represented by first degree formulae.

The musculature increases from about 20 per cent of the body weight in the smallest fetuses to a maximum of 33 per cent shortly before birth. It forms an average of a little less than half of the body weight in the adults. The locomotor system starts at about 40 per cent of the body weight in the early fetal period, rises to a maximum of 45 per cent, averages 41.5 per cent in the newborn cats and averages about 63 per cent of the body weight in the adults.

The muscles are about 1.3 times the weight of the skeleton in the smallest fetuses. This ratio increases to 2.7 before birth, then decreases to 2.4 in the early newborns. In the smallest adult males it starts at 2.5 and increases with the size of the cat to 4.3. The ratio is a little higher in the females and likewise increases with the weight of the cat.

The correlations of the weight of the musculature with the body weight, body length and with many of the organs average higher in the males. All of the correlations are positive in both sexes but several are too low to be significant.

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PHYSICAL ACTIVITIES AND RESPIRATION IN SLIME MOLDS

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In the course of a study on the effects of anesthetic agents on protoplasm, the senior author (1) observed that ethylene, a gas now much used in surgical anesthesia, had little or no noticeable anesthetic effect on the protoplasm of a myxomycete. Certain other gases proved to be excellent anesthetic agents for this lowly organism; such were carbon dioxide and cyclopropane, the latter a recognized anesthetic agent for human beings. Two further observations made during these earlier studies indicated that not only did ethylene have no anesthetic effect on slime molds, but it stimulated growth. More remarkable still was the fact that protoplasmic streaming continued when the concentration of ethylene administered was near 100 per cent. In an atmosphere of ethylene containing less than a half per cent oxygen, protoplasmic flow showed no signs of abatement, thus suggesting that the slime mold is anaerobic. These observations were the basis of the further study which is the subject of this paper.

Some preliminary data on respiration reported by the senior author (2) revealed an extraordinarily high oxygen consumption in the normal respiration of slime molds. This would appear to contradict the assumption of anaerobism. In the earlier experiments it was impossible to be certain that no oxygen remained within the moist chamber when ethylene was administered, but the oxygen present could not have exceeded 0.5 per cent. Subsequent work in which the chamber atmosphere was under more accurate control substantiated both of the earlier findings. It revealed a very high normal oxygen consumption, a coefficient of over 20, yet streaming continued until the oxygen supply was lowered to less than 0.5 per cent. Not until the concentration of oxygen was cut to 0.3 per cent was the streaming stopped, and then possibly for reasons other than low oxygen tension, e.g., possibly because of increased acidity. These observations were sufficient to indicate that the original suggestion of an anaerobic organ-

ism was in part correct. The assumption was based not on oxygen consumption, which later was shown to be very high, but solely on the fact that streaming continued under an oxygen pressure closely approaching zero.

It is reasonable to assume that respiration is the source of energy for protoplasmic movement. Consequently, if streaming continues in an atmosphere of extremely low oxygen tension, respiration must be anaerobic, or nearly so. The assumption proved to be at least half true, for it was shown that at a certain minimum oxygen pressure, respiration of one kind involving oxygen consumption, is reduced to very nearly zero, whereas respiration of another kind which is the source of energy for streaming, is in no way affected. These results can only be interpreted in terms of a dual respiratory system.

An incidental observation, which may explain some discrepancies, revealed that if the supply of oxygen is suddenly reduced, streaming may stop temporarily as a result of shock; sudden immersion in water will do the same. This is a form of shock anesthesia.

With this knowledge in hand the present authors undertook to examine: (*a*) the prediction already made, that ethylene, which anesthetizes man and is known to have a damaging effect on higher plants, actually stimulates the growth of slime molds in suitable concentration; and (*b*) if protoplasmic streaming in slime molds is anaerobic how may this be correlated with high oxygen consumption.

Material and Method

The living material worked upon in the experiments here reported was the myxomycete, *Physarum polycephalum*. The body of the plant, or animal, for the myxomycetes are also known as mycetozoa, is a plasmodium, a non-cellular sheet or net-like structure with many minute nuclei, much included matter such as fat globules, and a dense yellow pigment. The protoplasm is in a continuous state of flow, with a remarkable rhythmic reversal in direction of movement, reversal occurring on an average every three-quarters of a minute. When flow ceases naturally, the plasmodium is either dead or in a state of prolonged rest. The latter is the so-called sclerotial stage. When, because of experimental treatment, the protoplasm ceases streaming but later resumes its flow, it is in a state of anesthesia. The presence or absence of flow and its rate are good indicators of the state of the protoplasm.

Since molds are readily grown on moist paper if fed crushed oats, a method of culture now well known, for the slime mold Physarum is coming into use for many physiological studies (3). For experimental work a small portion of the plasmodium, about 0.5 g, is transplanted from the stock culture on to a glass slide which has been dipped into a solution of 2 per cent nutrition-free agar. The transplant soon spreads into a thin oval sheet which within an hour develops into a branched network with an irregular advancing periphery.

The rate of spreading may be determined by inverting an agar-covered slide carrying a small piece of plasmodium onto a microchamber and sealed with heavy vaseline. The gas chamber has stopcocks for controlling the entrance and exit of gas. The chamber is put into an apparatus resembling a large camera lucida which throws a magnified image of the culture onto a screen. The outline of the culture can thus be traced. The area of such tracings, made at fixed intervals, are ascertained with the aid of a planimeter, and the increase per unit time plotted.

The gases used were the purest commercial obtainable and came in pressure tanks. When the culture is to be subjected to a gas mixture, previous mixing in a constant pressure tank is useful. Such a tank consists of two glass vessels of large capacity, one of which is placed several feet above the other and connected with a siphon. The upper container when filled with water exerts a constant hydrostatic pressure on the gas in the lower tank. The latter is graduated to facilitate making up the gas mixture. A layer of inert mineral oil is interposed between the water and the gas in the lower vessel to insure against contamination. The gas from the lower vessel is led through a bubble counter into the gas chamber. The flow of gas, once adjusted, is constant. The gas-containing vessel is filled from the commercial tanks, the pressure of which forces the water out of the vessel. The rate of flow was adjusted usually to eight bubbles or 2 c.c. per second. The following gases and concentrations were used: a 100 per cent concentration of the unsaturated hydrocarbon ethylene; ethylene and oxygen in the proportions, 90-10, 85-15, 75-25, 70-30, 65-35, 50-50, 25-75; 100 per cent nitrogen; nitrogen and oxygen in the proportions, 25-75, 50-50, 75-25, 90-10, 95-5, 97-3. For comparison, a solution of 1/1000n KCN was applied directly to the culture by means of a fine pipette. In all cases an initial minimum time of one-half hour

was allowed to give the transplanted plasmodium time to commence normal spreading. The cultures were microscopically observed during each experiment to make certain that their condition was normal.

Before considering the experimental results it is necessary to decide how much if any of the increase in area of a newly made transplant is growth, and what are the distinctions between growth, locomotion, and similar movements. There are four forms of motion in a slime mold, all closely associated, but not necessarily interdependent; they are, growth, which involves an increase in mass; locomotion, which involves change in form but not necessarily an increase in area; spreading, which involves an increase in area but not necessarily growth; and streaming which may be involved in the foregoing three activities, but may also continue when growth, locomotion, and spreading are not present. Spreading, certainly most if not all of it, is simply a displacement of the protoplasm. It may involve some growth, but until this is proved it is better to regard the increase in area of a small transplant on nutrition-free agar as simply protoplasmic displacement, and refer to it as spreading, not growth. The spreading is, however, not to be regarded as the mere flattening out of a pile of semi-fluid matter. That this is true is shown by the fact that the rate of spreading is often faster in small bits than in larger ones, by the fact that during the first hour the rate of spreading increases with time, i.e., as the pile becomes smaller and by the very obvious fact that as the culture is on the underside of an inverted cover slip the spreading of the mass is against gravity. After all, we are dealing with organized living matter and not a drop of water. Spreading is accomplished by protoplasmic flow, but surface tension changes play a part, an increase in tension opposing protoplasmic pressure, and a decrease in tension assisting the pressure from within the organism.

Experimental Findings

1. *Normal Spreading*

When the respective areas of quarter-hour tracings of a spreading plasmodium are plotted against time, the graph is a straight line in more than 50 cases of normal spreading (Figure 3).

An interesting discovery to be discussed in a subsequent paper is the grouping into classes of spreading rates regarded as numerical patterns; each is an integral multiple of the smallest one.

FIGURE 1

Successive quarter-hour outlines of a spreading plasmodium showing three periods: 1. the increase in area during quarter-hour periods of normal spreading (1 to 3), 2. a half-hour period of no spreading with a slight change in contour, indicated by the time elapsing between contour line 3 and contour line 4 during which there was a constant flow of undiluted ethylene, and 3. resumption of normal spreading on return to an atmosphere of air (4 to 8).

2. *Ethylene*

When an undiluted stream of ethylene is allowed to flow through the gas chamber containing a plasmodium, the hitherto normal spreading is stopped and the outline of the culture remains practically unchanged for the duration of ethylene flow. On the removal of the ethylene normal spreading is resumed (Figure 1). Ordinarily no injury or disturbance of the protoplasm or change in streaming rate and rhythm could be discerned during the cessation of spreading. In some instances the plasmodium contracted slightly at the onset of the treatment and then remained unchanged. Occasionally, a subculture proved extremely sensitive to ethylene and developed islands of injury which invalidated the experiment. Slime mold protoplasm shows variability in behavior, one culture differing from another, and from day to day, as do all living organisms. Living matter possesses individuality which manifests itself in the most lowly forms of life.

3. *Nitrogen*

The action of an undiluted stream of pure nitrogen is precisely the same as that of ethylene except for the absence of injury, which occasionally shows up in ethylene treated protoplasm, and the lack of any stimulation to spreading such as ethylene rather regularly produces at proper concentrations. Therefore, the effect of ethylene, with the foregoing two exceptions, is due not to ethylene as such but to the absence of oxygen.

In order to be certain of an oxygen free supply of nitrogen, the commercial N_2, containing 0.005 per cent O_2, was passed over very hot copper. The N_2 thus purified was cooled to room temperature and passed into the moist chamber containing the slime mold. Protoplasmic flow continued for twenty minutes then ceased. The N_2 supply was replaced by air, and in 10 minutes movement was observed with rapid recovery to fully normal protoplasm. No injury of any kind was to be seen.

The similarity in the effects of both ethylene and nitrogen on protoplasm refers only to inhibition of spreading while streaming continues.

If rate of spreading, unity being taken as normal, is plotted as ordinates against oxygen tension in per cent as abscissae the curve rises

rapidly from zero spreading at zero oxygen tension to full normal spreading (unity) at 10 per cent oxygen, and continues normal from there on (Figure 2).

The fact that the inhibition of the spreading mechanism of plasmodia by non-toxic, undiluted nitrogen and ethylene is due to the exclusion of oxygen indicates that there are present at least two distinct energy providing systems, one for spreading and locomotion, and probably also for growth, and one for streaming. As a general concept, this idea is not new. In the present case, the mechanism-providing energy for spreading and growth is a respiratory system consuming large amounts of oxygen, whereas the energy for streaming is supplied by a feebly aerobic respiratory system if not by anaerobic fermentation. This assumption is corroborated not only by the studies of the senior author and Price (2) but also by later unpublished work with *KNC* by Price and Allen of this laboratory.

4. *Potassium Cyanide*

KCN in 1/1000 concentration stops spreading completely with no visible effect on streaming. Though spreading stops, locomotion continues, i.e., the shape of the plasmodium changes without increase in area.

The effect of *KCN* on respiration is pronounced. Respiration is inhibited to a large extent, but protoplasmic flow continues. Azide, on the other hand, inhibits streaming reversibly. These two facts indicate the presence of a special insensitive mechanism to cyanide, and of very low oxygen consumption, 0.3 per cent oxygen being sufficient to supply the energy for streaming.

The *KCN* sensitivity of one of the energy producing mechanisms presented us with another opportunity to test the probability of the two system theory, and the possible dependence of spreading on respiration. If *KCN* of *N*/1000 concentration inhibits 80 per cent of the respiration, spreading should be cut severely when *KCN* is applied. This is indeed the case, spreading ceases immediately on application of *KCN*. Recalling, now, that oxygen consumption is high and that streaming is not retarded until the oxygen supply is reduced to 0.3 per cent, the presence of two systems is a justifiable assumption. One of them, a respiratory system of high oxygen consumption, supplies the mechanisms responsible for growth, spreading, and loco-

motion and is cyanide sensitive; the other, of extremely low oxygen consumption, supplies the streaming alone.

5. *The Stimulating Effect of Ethylene*

In the earlier work herein referred to, a stimulation of growth had been observed when a slime mold culture is subjected to dilute ethylene. Contrary to expectations, there was no anesthesia, no cessation of streaming, ordinarily no injury, but actually a stimulation to growth at a concentration of ethylene of three parts in four or four parts in five, a concentration which gives the maximum of anesthetic effect yet leaves ample oxygen for respiration.

This earlier work was repeated in order to substantiate it and correlate it with subsequent findings.

The results were gratifying. In 75 per cent ethylene, which includes ample oxygen for normal respiration, there is an immediate

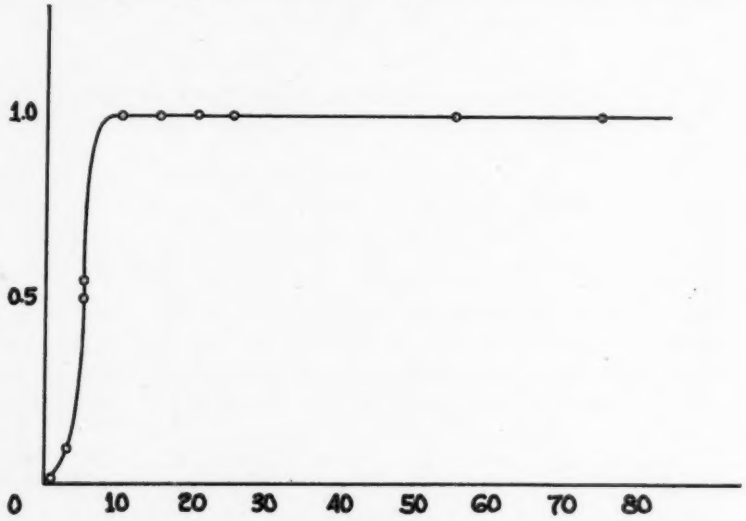

FIGURE 2

The effect of oxygen tension on spreading. Rates of spreading as ordinates are plotted against O_2 tension in per cent as abscissae. The base line is zero spreading, and unity is normal spreading; the latter is resumed at 10 per cent oxygen. The oxygen is diluted with N_2.

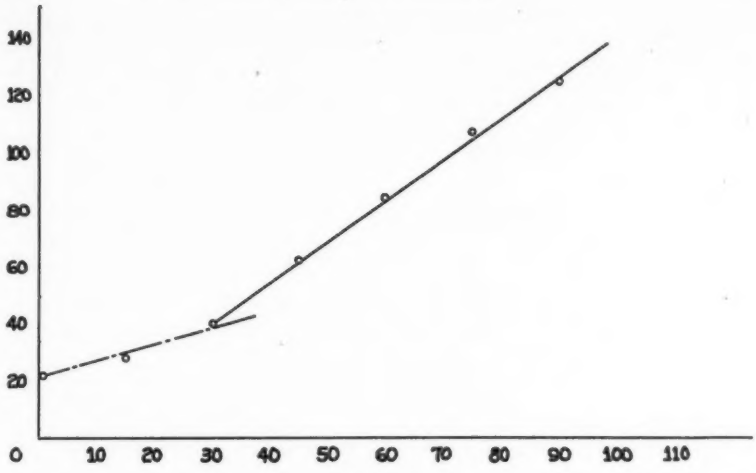

FIGURE 3

Normal rate of spreading (broken line) and accelerated rate of spreading (solid line) due to ethylene at 75 per cent concentration (with 25 per cent O_2. Spreading in terms of areas in sq. cm. are plotted as ordinates against time in minutes as abscissae. Note that both graphs are straight lines.

increase in spreading, in area covered per 15 minute period (Figure 3). Two facts are to be emphasized here, that both normal spreading in air and accelerated spreading in diluted ethylene, plotted against time, yield straight lines (Figure 3), and that with increase in concentration of ethylene, to a maximum at 75 or 80 per cent ethylene, there is a gradual increase in the rate of spreading, followed by a fall to zero spreading at near 100 per cent (Figure 4).

There can be no question then that ethylene is a stimulant to growth at the rate of increase in bulk when food is added, and also to spreading or increase in area in cultures free from nutrition. The average maximum increase in the rate of spreading of slime molds due to ethylene is 2.5x.

When, on the other hand, similar concentrations of nitrogen and oxygen are applied, no stimulation occurs. The spreading rate remains normal provided five or more per cent oxygen are present. Below 5 per cent oxygen the spreading rate falls rapidly to zero. The total absence of oxygen stops streaming in a few minutes if suddenly ap-

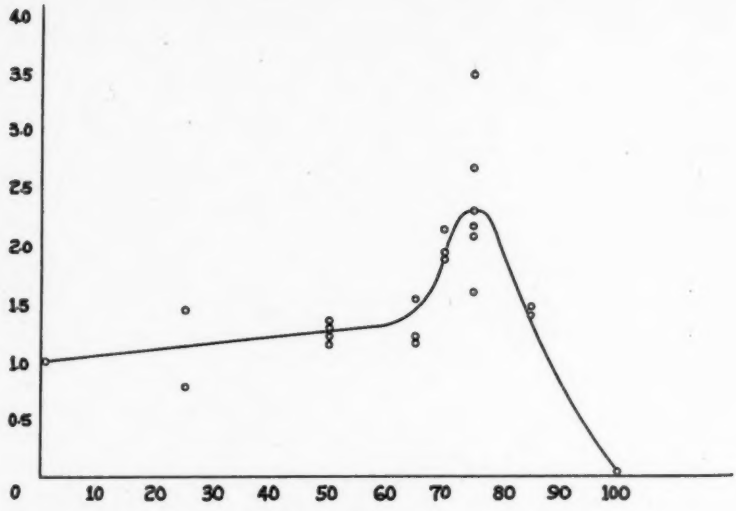

FIGURE 4

Effect of ethylene on rate of spreading. Concentrations are from 0 to 100 per cent ethylene with O_2. Ordinates are rates of spreading; abscissae are per cent ethylene. Maximum acceleration of spreading is between 75 and 80 per cent ethylene.

plied. It is our opinion now that if oxygen pressure is very gradually reduced to zero, streaming will continue, certainly to very near zero O_2 pressure.

Discussion

Several of the findings of this research will be considered in subsequent publications. One such is the fact that the graph of normal spreading obtained by plotting total area against time is a straight line (Figure 3). At the moment our major problem is the startling discovery that spreading and locomotion may be stopped without any inhibition or streaming. Streaming has long been considered the mechanism of amoeboid movement. The senior author recently took occasion (2) to sharply distinguish between streaming and locomotion. Vouk (4) has stated that the advancement of a plasmodium in a specific direction is independent of the flow of the protoplasm in that direction.

The spreading of a small transplanted mass of plasmodium into a

round sheet is a combination of thinning, locomotion, and on occasion growth. The four functions, streaming, spreading, locomotion, and growth are intimately associated in a slime mold, yet it appears from this work that they are not inseparable.

As the experiments here recorded show that streaming may continue actively, though spreading, locomotion, and growth are at a standstill, it would appear that the primary function of streaming is circulation, only secondarily is it an aid to locomotion. Consequently, streaming is principally concerned with the distribution of oxygen and food, and the elimination of waste products. This statement does not deny that locomotion and growth in slime molds, and perhaps in some other organisms, depend on streaming; locomotion unquestionably does, but protoplasmic flow may continue when locomotion and enlargement, both as spreading and growth, cease, indeed, it must continue in order to fulfill other primary functions.

A very significant feature of the total respiratory mechanism of an organism, which the present work supports, is the ability of an organism to apply the last energy reserve still available in any drastic situation, to that mechanism which among all the processes necessary for prolonged living is absolutely essential to life. In other words, if the total oxygen supply necessary to complete living is gradually reduced, the organism first gives up those functions needing oxygen which are the least essential. This means that under conditions greatly reducing the total oxygen needed to keep the main respiratory system going, those functions which can be temporarily dispersed with are first cut. With the very small amount of energy still available the plasmodium is unable to move or grow, but so essential a process as streaming, with its minimal oxygen need, can still carry on.

The whole question may be viewed in the light of the relative importance to the organism of the several functions. If energy, derived from different sources, is distributed to consuming processes, the relatively more important ones will be kept going in preference to the relatively less important ones if the total energy accessible is cut. Streaming, which serves primarily in moving food, supplying oxygen, and taking away waste products, may be regarded as a greater need than either locomotion or growth, which in the slime molds also depends indirectly on streaming.

We may go further and attempt a comparison of the relative importance of growth and locomotion. An observation which makes this possible was made on a slime mold specimen under the influence of *KCN*. Under these conditions respiration is cut 70 per cent and spreading is completely stopped; the total area does not change, but the outline of the plasmodium is constantly changing. This means that although there is not actual spreading, i.e., no increase in area, there is locomotion or change in shape. This supports the above mentioned hypothesis, that one function, locomotion, is more necessary to the slime mold than certain others, such as spreading or growth, an assumption which seems quite plausible as myxomycetes depend on locomotion as a means for finding food. The relative importance of the phenomena under consideration would then be, in decreasing order: streaming, locomotion, spreading, and growth.

It is exceedingly difficult to be sure that a moist atmosphere, such as a slime mold must have in order to survive, is wholly free of oxygen. For this reason we have frequently referred to an atmosphere as "nearly" devoid of oxygen, "nearly" 100 per cent ethylene or nitrogen. As an atmosphere wholly free of oxygen is approached, a slime mold shows signs of injury and streaming must stop, but this atmosphere must contain less than 0.3 per cent oxygen. Also, we have suggested that if oxygen pressure is very gradually reduced to zero, streaming may continue. Streaming can be stopped by shock, so mild a shock as plunging in water being sufficient. But there is still another possibility which our co-worker, Jerome Zwanger, has corroborated. If a slime mold plasmodium is bathed in a suitable buffer (Na acetate-acetic acid) acute anoxia in 100 per cent nitrogen is avoided. This may be interpreted in terms of a mechanism in which excess acids are produced as a result of an abnormal environment and a disturbed metabolism. If a buffer is added which will take up excess acids as rapidly as they are formed, streaming continues in spite of greatly reduced oxygen, even below 0.3 per cent.

Summary

1. A method of studying and measuring the rate of spreading of the plasmodia of myxomycetes is described.

2. Normal spreading gives a straight line graph, indicating that the spreading goes out from one or a few localized centers.

3. At very low oxygen tension all spreading or increase in area is inhibited, but streaming is not affected.

4. Ethylene increases the rate of both growth and spreading in all concentrations up to 85 per cent.

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